# TOXICITY OF MERCURIC CHLORIDE IN CULTURES OF NEURONS AND NON-NEURONAL CELLS DERIVED FROM EMBRYONIC CHICK SYMPATHETIC GANGLIA

by

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THE UNIVERSITY OF UTAH GRADUATE SCHOOL

# SUPERVISORY COMMITTEE APPROVAL

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## ABSTRACT

The effects of mercuric chloride on both mixed and highly purified cultures of neurons and non-neuronal (glial) cells were studied. Cultures were prepared from the sympathetic ganglia of 11 day chick embryos and treated with mercuric chloride (0.0001-100 μM) for two or three days. Protein content, acetyl cholinesterase activity, $^{3}$H-thymidine incorporation, and $^{14}$C-leucine incorporation were measured, and the following results were obtained. First, the effects of mercuric chloride varied in a complex manner with both concentration and time of exposure. For example, mixed cultures treated for two days showed (a) increased total protein content and $^{14}$C-leucine incorporation at low concentrations, (b) decreased total protein content and $^{14}$C-leucine incorporation at high concentrations, (c) increased acetyl cholinesterase activity at most concentrations, and (d) decreased $^{3}$H-thymidine incorporation at the highest concentration. In cultures treated for three days, essentially all concentrations of mercuric chloride either inhibited or had no effect on any of these biochemical quantities. Second, neurons were sensitive to concentrations of mercuric chloride as low as 0.0001-0.001 μM while the non-neuronal cells were generally unaffected by

concentrations less than 1 μM. Third, most of the effects of mercuric chloride resulted from direct actions on either the neurons or non-neuronal cells. For example, various concentrations of mercuric chloride had identical effects on the activity of the neuronal enzyme acetyl cholinesterase in the presence and absence of non-neuronal cells (i.e., in mixed and highly purified neuronal cultures, respectively).

## ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

# INTRODUCTION

Mercury is now widely recognized as an extremely hazadous environmental pollutant because of the occurences of mercury poisoning in Japan in the 1950's and in Iraq in the 1970's. The clinical signs and symtoms of Minamata disease involve a wide variety of neurological disturbances including impairment of vision and other sensory inputs, speech and hearing defects, ataxia, mental disturbances, etc. (Takeuchi et al.,1962). Common neuropathological changes observed in both human autopsy material and in the brains of treated animals include neuronal degeneration and glial cell proliferation (gliosis) (Chang and Hartmann, 1972b; Charbonneau et al., 1976; Chang, 1977).

A wide variety of different biochemical alterations in the brains of animals exposed to mercury compounds have been described. Intoxication of rats with mercuric chloride resulted in a decrease in the RNA content of neurons in the dorsal root ganglia and an increase in the RNA content of anterior horn motorneurons (Chang and Hartmann, 1972a). Other studies have demonstrated a change in the base composition of RNA in spinal ganglion neurons exposed to mercuric chloride for 11 weeks (Chang, 1977). Studies by Yoshino et al. (1966) and others (Verity et al., 1977;

Cavanagh and Chen, 1971) have shown that intoxication of rats with a mercury compound resulted in an early marked reduction in protein synthesis in the brain which preceded development of other neurological and biochemical changes. In contrast, Brubaker et al. (1973) found increased protein synthesis after mercury exposure. Numerous studies have also been carried out on the activity of specific enzymes following exposure to mercury compounds. Chang et al. (1973) found a general reduction in the activities of all brain enzymes examined, but Webb (1966) found that the activity of some enzymes increased while that of other enzymes decreased.

Mercury compounds have also been demonstrated to impair the function of the blood-brain barrier. For example, Chang and Hartmann (1972c) and Ware et al. (1974) have demonstrated that minute amounts of mercury can impair the blood-brain barrier within a few hours and allow entry into the brain of plasma components which are usually excluded from the nervous system. In addition, other investigators have demonstrated that mercury cause a great reduction in the ability of the blood-brain barrier to actively take up substrates such as amino acids. The extent to which these changes in the blood-brain barrier might indirectly affect the biochemistry of neurons and glia within the nervous system has not yet been determined.

Most of these biochemical studies have not attempted to answer the following fundamental questions with regard to the sites and mechanisms of action of mercury compounds. First, do the various biochemical changes which occur in mercury-intoxicated brains, take place in the neurons, in the glial cells, or both cell types? Even the elegant studies of Chang et al. (1972) on the RNA content of single neurons in mercury-intoxicated rats did not determine whether similar changes took place in the glial cells. Second, are observed changes in a given cell type always the result of a direct action of the mercury compound on the particular kind of cell? For example, the gliosis which results from mercury intoxication might occur because of neuronal degeneration caused by the mercury (Hanson and Partlow, 1977) rather than as a result of a direct action of the metallic salt on the glial cells. Answers to such questions can best be obtained by use of simplified in vitro systems.

One advantageous system for the study of the effects of heavy metals on nervous tissue utilizes essentially pure ($\geq$ 99%) primary cultures of separated neurons and non-neuronal (glial) cells derived from embryonic chick sympathetic ganglia (McCarthy and Partlow, 1976a). The non-neuronal cells in these cultures appear to be either satellite cells or Schwann cells, both of which are glial elements, while the neurons are postganglionic sympathetic neurons. Since mixed cultures of neurons and non-neuronal cells can also be

prepared (McCarthy and Partlow, 1976a), it is possible to compare directly the effect of heavy metals on the biochemistry and morphology of (1) neuronal, (2) non-neuronal, and (3) mixed cultures. Utilization of this system should make it possible to determine the extent to which some of the metabolic effects which have been observed in vivo are due to direct actions on either the neurons or glial cells in the nervous system.

The studies to be presented in this paper demonstrate that the responses of neurons and non-neuronal cells to various concentrations of mercuric chloride are quite complex. By appropriate selection of concentration and time of exposure, mercuric chloride could be shown to either stimulate, inhibit, or have no effect on any of four biochemical quantities (protein content, acetyl cholinesterase activity, $^{14}$C-leucine incorporation, and $^{3}$H-thymidine incorporation). Neurons were found to be much more sensitive to mercuric chloride than the non-neuronal cells. In most cases the effects of mercuric chloride observed in mixed cultures containing both neurons and non-neuronal cells were ascribable to direct actions on either the neurons or non-neuronal cells. However, in several situations, the effects of mercury on mixed cultures were quite different from those observed in highly purified neuronal and non-neuronal cultures.

## MATERIALS AND METHODS

Culture medium and salt solutions. L-15 (Leibovitz) Medium (North American Biologicals, Miami, Fla.) was supplemented ($L-15^{+}$) so that the final solution contained 10% fetal calf serum (Irvine Scientific, Fountain Valley, Calif.), 0.5% glucose (analytical reagent, Mallinckrodt, Saint Louis, Mo.) 100 I.U./ml Penicillin, and 100 µg/ml streptomycin (Grand Island, Biological Co., Grand Island, N.Y.). The fetal calf serum was equilibrated with air before addition to the culture medium by stirring it for two hours at room temperature. This medium was then adjusted to 300 mOsM and a pH of 7.15 at 37°C. Nerve growth factor (Burroughs Welcome Co., Research Triangle Park, N.C.) was added to all cultures containing sympathetic neurons to make a final concentration of 15 ng/ml. Puck's saline "G" without phenol red (Irvine Scientific) was supplemented with 5 mg/ml glucose.

Culture surfaces. Falcon plastic dishes, 35 mm in diameter, (Oxnard, Calif.) were used in all experiments. Both mixed cultures and highly purified non-neuronal cultures were grown directly on the polystyrene surface of these dishes. For reasons described by Wallace and Partlow (1978), all highly purified neuronal cultures

were grown in polylysine-coated dishes which were prepared according to the procedure of Letourneau (1975). One ml of 1 mg/ml polylysine (Nutritional Biochemical Corp., Irvine, Calif.) in pH 8.4 borate buffer solution was added to each dish. The dishes were then incubated overnight at 37°C, the polylysine solution was removed, and the dishes were rinsed five times with sterilized deionized water before use.

Culture wells. Pyrex tubing with inner diameter of 13 mm was cut into glass cylinders approximately 5 mm tall. All glass cylinders were boiled in 0.1 M potassium hydroxide, rinsed extensively with water, and autoclaved before use. The cylinders were sealed to the inner surface of culture dishes with Dow Corning high vacuum grease (Midland, Mi.).

Cultures. The thoracolumbar paravertebral ganglia from 11 day chick embryos provided the material for all cultures. Both mixed ganglion cell cultures and highly purified neuronal and non-neuronal cultures were prepared according to the method of McCarthy and Partlow (1976a). Both the number of cells ( $2 \times 10^5$ cells/well) and the volume of medium (200µl) were constant in all experiments. Mixed cultures grown under these conditions were actively dividing during 6 days of growth in vitro (Table 1). A set of "blank" culture wells containing culture medium but lacking cells was included in each experiment to serve as controls for all

Table 1. $^{3}$H-Thymidine incorporation by mixed sympathetic ganglion cell cultures grown for up to six days *in vitro*[+].

| Incubation Period (days) | $^{3}$H-Thymidine Incorporation (DPM/culture) |
|---|---|
| 2 | 9,542 ± 361 (4)[#] |
| 4 | 15,231 ± 464 (4)[#] |
| 6 | 12,047 ± 579 (4)[#] |

[+] Mixed cultures were prepared from the sympathetic ganglia of 11-day chick embroys and incubated in L-15[+] culture medium as described in Materials and Methods. $^{3}$H-Thymidine was added to selected cultures after 28, 76, or 124 hours *in vitro* and the cultures were incubated for an additional 20 hours. All cultures were harvested by sonication and assayed for incorporation of $^{3}$H-thymidine into acid-precipitable macromolecules (see Materials and Methods).

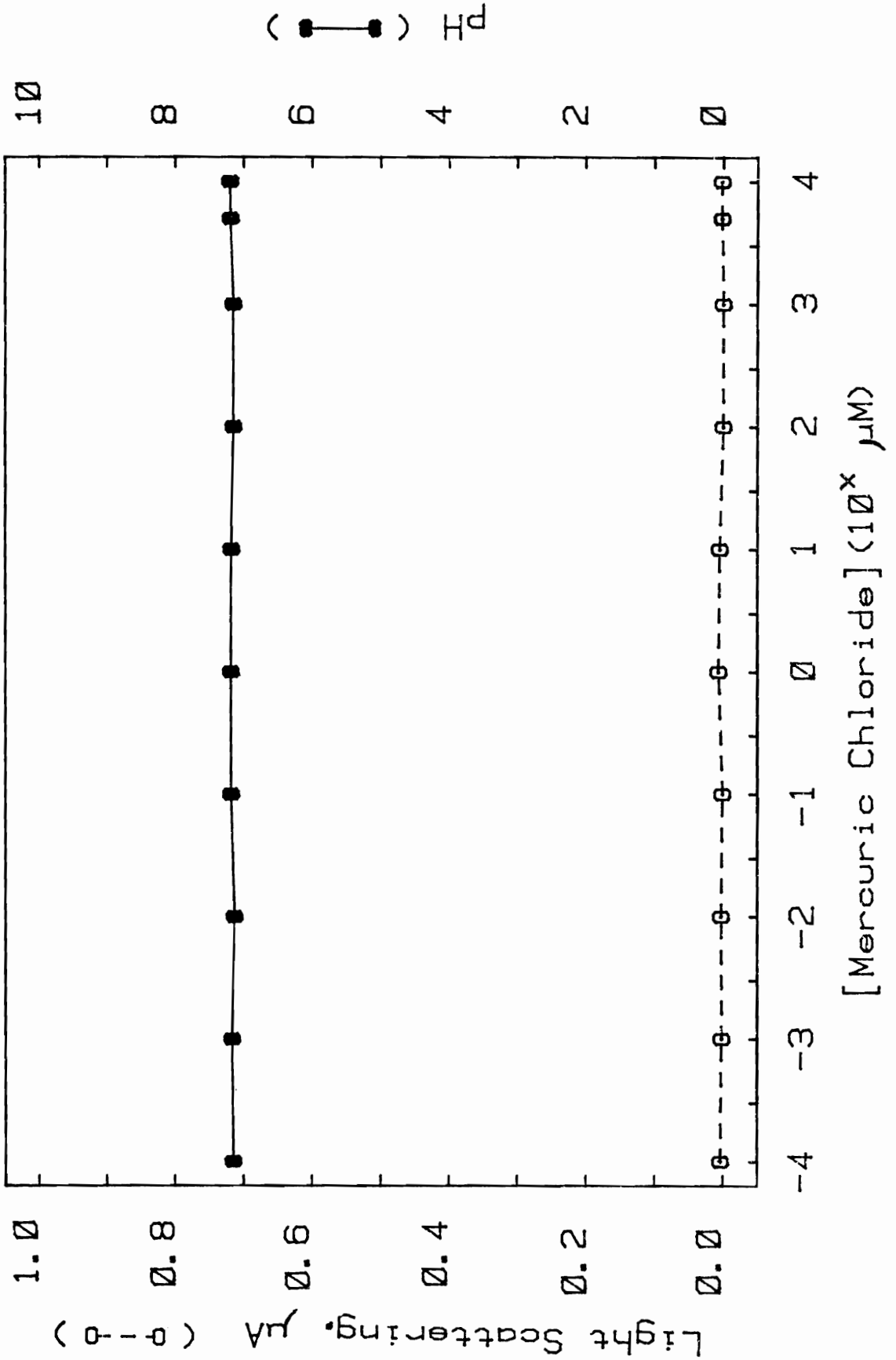
[#] Each of these values is significantly different from both other values ($p \geq 0.01$). The number of observations (n) is given in parenthesis.

assays. These blank preparations were incubated and harvested along with the cell-containing cultures.

Metal treatment. $L\text{-}15^+$ culture medium containing various concentrations of mercuric chloride (Mallinckrodt, analytical reagent) was prepared by addition of a constant proportion (1:19) of a solution of the metallic salt in sterile deionized water. Culture medium for untreated control cultures was diluted to the same extent. Addition of mercuric chloride to the $L\text{-}15^+$ culture medium to make final concentrations of 0.1 nM to 10 mM had no effect on either the pH of the medium or the amount of precipitated material in the medium as measured by light scattering (Fig. 1).

In all experiments, the cultures were incubated in $L\text{-}15^+$ medium lacking heavy metal for 24 hr in order to allow time for the cells to attach and begin growing (Fig. 2). The normal medium was then removed and replaced with $L\text{-}15^+$ medium containing from 0.0001 to 100 μM mercuric chloride (Fig. 2), except that the medium bathing the untreated control cultures never contained mercuric chloride. In all cases, the medium was changed every 24 hr (Fig. 2). $L\text{-}15^+$ medium containing mercuric chloride bathed the cultures for either 2 days (Fig. 2A,C) or 3 days (Fig. 2B). In some experiments, the medium containing the metallic salt was removed after the cultures were exposed to the heavy metal for two days and the cells were allowed to recover (Fig. 2C). In

Figure 1. Lack of an effect of mercuric chloride on either the pH of L-15$^{+}$ tissue culture medium or the solubility of its constituents as measured by light scattering. Aliquots of L-15$^{+}$ medium containing various final concentrations of mercuric chloride were prepared as described in Materials and Methods and incubated at room temperature for 1 hr. Light scattering was measured by use of a Farrand fluorometer (Wallace and Partlow, 1978); values are given in microamperes generated by the photomultiplier tube. pH was determined by use of a Model 39030 Beckman combination electrode and a Corning Model 12 pH meter. Each point represents the mean of three measurements.


Light Scattering, μA (o--o)
1.0
0.8
0.6
0.4
0.2
0.0
10
8
6
4
2
0
pH (●—●)
-4
-3
-2
-1
0
1
2
3
4
[Mercuric Chloride] (10^x μM)

these experiments a large volume of L-15$^{+}$ medium (3 x 0.4 ml) was used to rinse away as much of the metallic salt as possible and fresh medium lacking mercuric chloride was then added.

Incorporation of $^{3}$H-thymidine and $^{14}$C-leucine. [Methyl-$^{3}$H]-thymidine (New England Nuclear, Boston, Mass., 6.7 Ci/mmole; 0.05 μCi/culture) and L-[$^{14}$C(U)]-leucine (New England Nuclear, 325 mCi/mmole; 0.05 μCi/culture) were added to all cultures either 24 hr (Fig. 2A) or 48 hr (Fig. 2B, C) after the addition of culture medium containing mercuric chloride, and the incubation was continued for 20 hr before harvesting. Amounts of $^{3}$H-thymidine and $^{14}$C-leucine incorporated into acid-precipitable macromolecules were determined by a paper disc method as described by Partlow and Larrabee (1971).

Cell harvesting procedure. Medium from each culture well was removed, and the attached cells were rinsed 3 times with 0.4 ml of Puck's saline "G" to remove as much as possible of the unincorporated labels. The cells were harvested by sonication in ice-cold distilled water (2 x 60 μl). The pair of aliquots corresponding to each culture sonicate was pooled in a 0.4 ml microcentirfuge tube (Arthur H. Thomas Co., Philadelphia, Pa.) and this combined sonicate was mixed thoroughly. A portion of each combined sonicate was set aside for assay of acetyl cholinesterase in another microcentrifuge tube containing a sufficient amount

of bovine serum albumin (Sigma Chemical Co., Saint Louis, Mo.) to yield a final concentration of 0.1%. The microcentrifuge tube containing the combined sonicate without albumin was used for both protein analysis by the technique of Lowry et al. (1951) and for analysis of incorporated $^{3}$H-thymidine and $^{14}$C-leucine. Both portions of the combined cell sonicate were capped and store at -80$^{o}$C until analysis.

Acetyl cholinesterase assay. Acetyl cholinesterase (3.1.1.7 acetylcholine acetylhydrolase) activity was determined by the method of Ellman et al. (1961) using acetylthiocholine (Sigma Chemical Co., Saint Louis, Mo.) as a substrate. It was not necessary to use inhibitors of butyryl cholinesterase (3.1.1.8 acetylcholine acylhydrolase), since this enzyme is not present in either sympathetic neurons or non-neuronal cells (McCarthy and Partlow, 1976a). Since true acetyl cholinesterase is only found in neurons in these mixed cultures (McCarthy and Partlow, 1976a), it was used as a neuronal marker.

Statistics. All measurements reported in this paper are given as means plus and minus the standard errors of the means. The number of observations (n) is given in parentheses. Differences between control and treated means were evaluated by

the Student "t" test and are marked in tables and figures with a single asterisk (*) if $0.25 \geq p > 0.01$. Differences between mean values are indicated by a pair of asterisks (**) if $p \leq 0.01$.

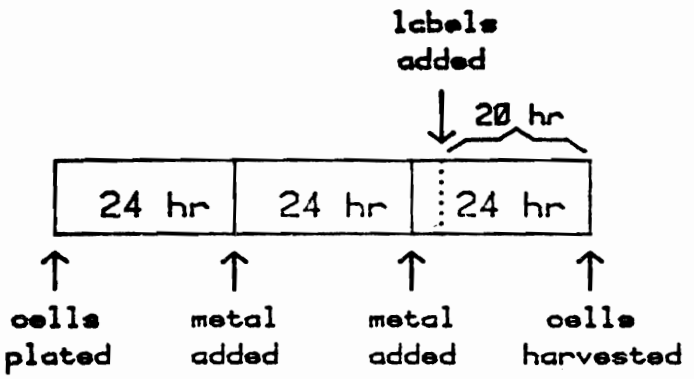
# RESULTS

Experimental design.

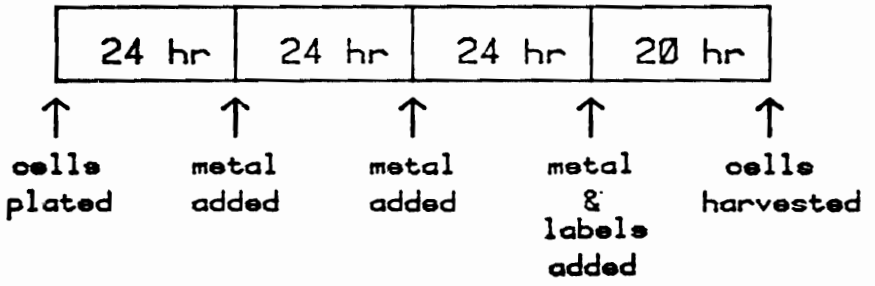
Three different experimental designs were employed in this study. In two day treatment experiments, sympathetic cultures were incubated for three days and exposed to various concentrations of mercuric chloride during the final two days *in vitro*. (Fig. 2A). In three day treatment experiments, cultures were incubated for four days and exposed to the metallic salt during the final three days (Fig. 2B). Finally, in a series of recovery experiments, cultures were incubated for four days but only exposed to mercuric chloride on the second and third days *in vitro* (Fig. 2C). In all cases, $^{3}$H-thymidine and $^{14}$C-leucine incorporation were measured during the final 20 hr of incubation (Fig. 2). This experimental design made it possible to compare the effects of both two days and three days of exposure to mercuric chloride on sympathetic ganglion cell cultures, and to determine the recovery capacity of these cultures.

Figure 2. Design of the three types of experiments utilized in this study. Sympathetic ganglion cells were prepared as described in Materials and Methods and plated at zero time. Medium containing various concentrations of mercuric chloride was used in place of the standard L-15$^{+}$ medium after 24 hr, 48 hr and, in some experiments (B), again after 72 hr ("metal added"). Medium containing mercuric chloride was therefore present for either two (A, C) or three days (B) in vitro. In all cases, $^{3}$H-thymidine and $^{14}$C-leucine were added ("labels added") 20 hr prior to termination of the experiment ("cells harvested").

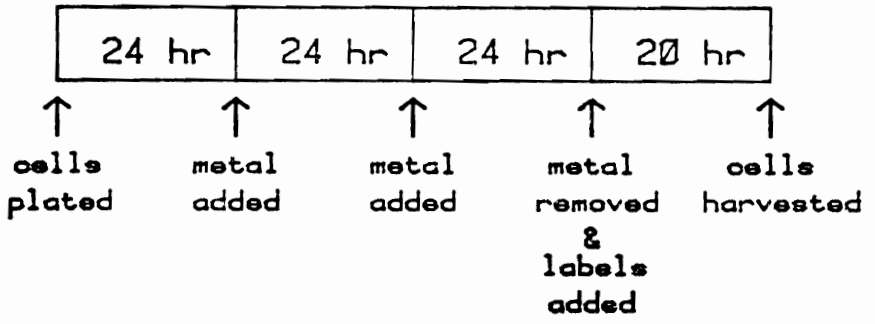
## A. Two-day Treatment




## B. Three-day Treatment




## C. Recovery Experiment



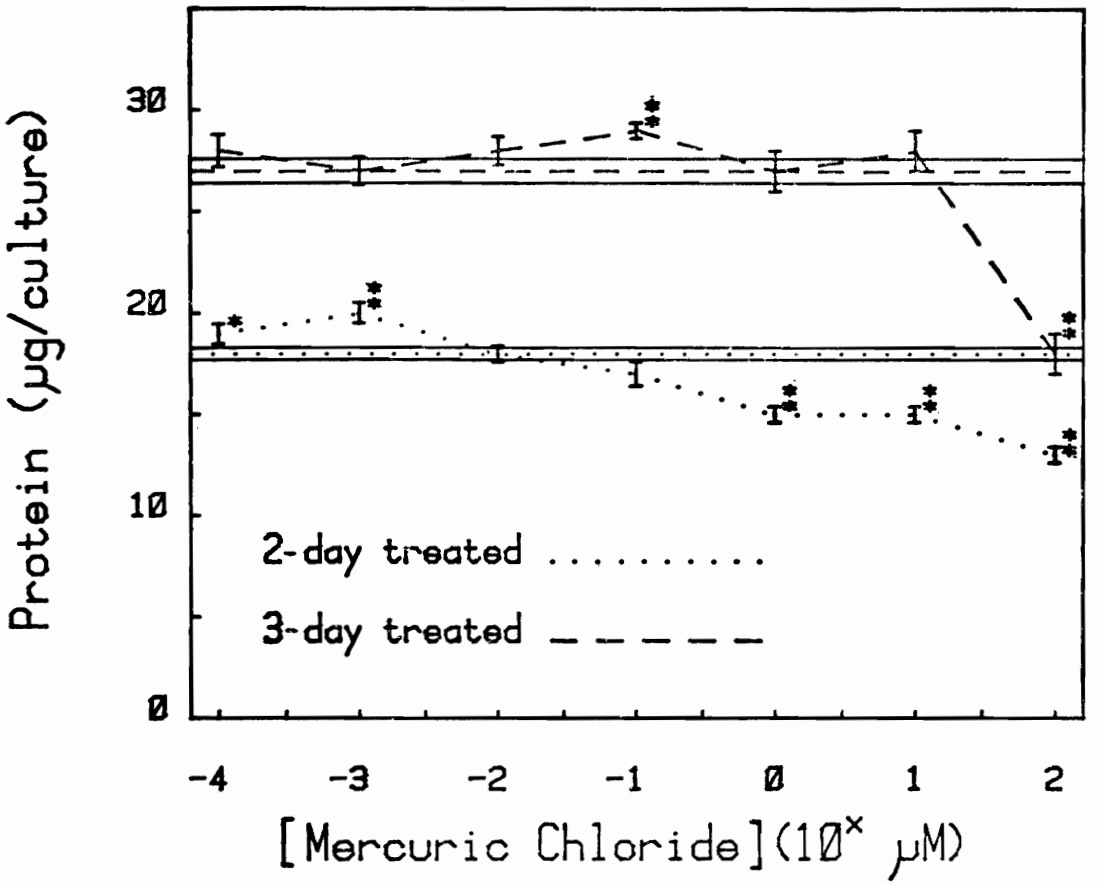
Effects of mercuric chloride on mixed sympathetic cultures treated for either two or three days.

Culture morphology. No obvious morphological effects were observable in either the neurons or non-neuronal cells treated for two or three days *in vitro* at any concentration of mercuric chloride from 0.0001 to 100 μM (pictures not shown). However, it did appear that somewhat greater amounts of cell debris were present in treated cultures than in untreated control cultures.

Protein content. The amount of protein in untreated control cultures increased from 18 ± 0.3 μg/culture (n = 6) after 72 hr to 27 ± 0.6 μg/culture (n = 6) after 92 hr *in vitro* (see control values in Fig. 3 corresponding to two- and three-day treatment experiments, respectively). Thus, the total amount of protein in these mixed cultures increased by 50% during the fourth day *in vitro*.

In mixed cultures treated with mercuric chloride for two days, a slight but significant increase in total protein content was observed at both 0.1 and 1.0 nM (Fig. 3). As the concentration of mercuric chloride was further increased, the total protein content tended to decrease. Significant decreases were observed at all concentrations equal to or greater than 1 μM.

Figure 3. Total protein content of mixed sympathetic ganglion cell cultures exposed for either two or three days to various concentrations of mercuric chloride. Mixed cultures were prepared from sympathetic ganglia of 11-day chick embryos, handled as described in Figure 2A, B, harvested by sonication, and assayed for protein as described in Materials and Methods. Values for untreated control cultures are shown as bars extending across the width of graph for both two- and three-day treatment experiments. All values represent means $\pm$ SE. Means of treated cultures which differ significantly from that of the matched control are marked with a single asterisk (*) if $0.025 \geq p > 0.01$ and with two asterisks (**) if $p \leq 0.01$

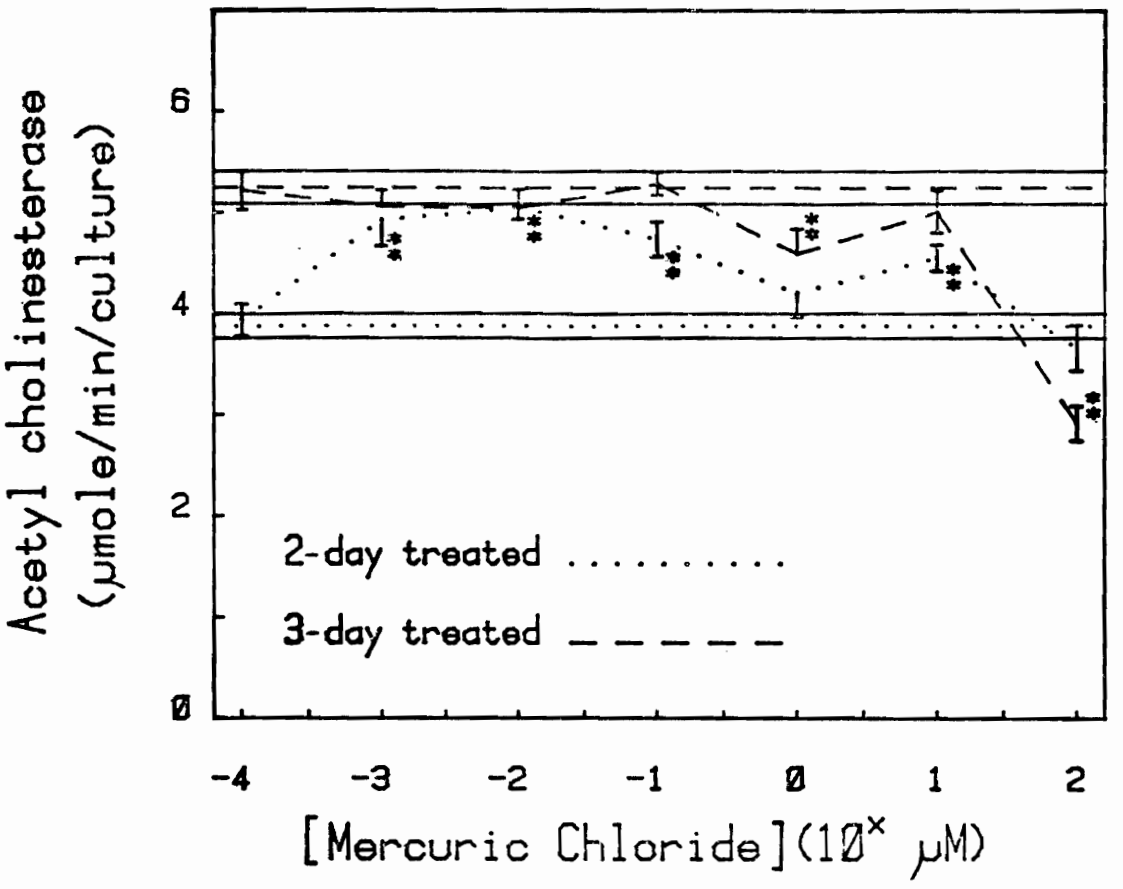

Protein (µg/culture)
30
20
10
0
2-day treated .....
3-day treated _ _ _ _ _
-4
-3
-2
-1
0
1
2
[Mercuric Chloride](10^x µM)

These decreases ranged from 14% at 1 μM to 31% at 100 μM.

In mixed ganglion cell cultures treated with mercuric chloride for three days, little effect on total protein content was observed at any concentration between 0.1 nM and 10 μM (Fig. 3). Thus, neither the stimulatory effects seen at 1 and 10 μM after two days of treatment were evident after three days of exposure (Fig. 3). In contrast, total protein was very slightly elevated at 0.1 μM in cultures treated for three days but not in cultures treated for two days (Fig. 3). Data obtained after treatment for either two or three days were similar in that 100 μM mercuric chloride caused a significant reduction in the total protein content of both groups of cultures. This decrease was only slightly greater after three days of treatment than that observed after two days of exposure (37% and 31%, respectively).

Acetyl cholinesterase activity. The amount of acetyl cholinesterase in untreated control cultures increased from $3.88 \pm 0.12$ μmol/min/culture (n = 6) after 72 hr to $5.24 \pm 0.16$ μmol/min/culture (n = 6) after 92 hr *in vitro* (see control values in Fig. 4 corresponding to two- and three-day treatment experiments, respectively). These results demonstrated that the activity of this neuronal marker enzyme increased by 35% during the fourth day *in vitro* in these mixed cultures.

**Figure 4.** Acetyl cholinesterase activity in mixed sympathetic ganglion cell cultures exposed for either two or three days to various concentrations of mercuric chloride. Mixed cultures were prepared from the sympathetic ganglia of 11-day chick embryos, handled as described in Figure 2A, B, harvested by sonication, and assayed for acetyl cholinesterase as described in Materials and Methods. All values represent means $\pm$ SE (n = 4-8). Values of untreated control cultures are shown as bars extending across the width of the graph for both two-and three-day treatment experiments. Means of the treated cultures which differ significantly from that of the matched control are marked with two asterisks (**) if $P \leq 0.01$


Acetyl cholinesterase
(μmole/min/culture)
6
4
2
0
2-day treated .....
3-day treated _ _ _ _ _
-4
-3
-2
-1
0
1
2
[Mercuric Chloride](10^x μM)

In mixed cultures treated with mercuric chloride for two days, acetyl cholinesterase activity was significantly increased at all concentrations between 1 and 100 nM and at 10 μM (Fig. 4). These data indicated that mercuric chloride can increase acetyl cholinesterase activity in cultured sympathetic neurons. This increase could result from (a) action on the enzyme, (b) a direct effect on the neurons to increase enzyme synthesis or (c) an indirect effect on the non-neuronal cells present in the mixed cultures. These possibilities were tested (vide infra).

No effect was seen in mixed sympathetic cultures treated for two days at either the lowest (0.1 nM) or the highest (100 μM) concentrations of mercuric chloride (Fig. 4). In addition, no effect was found in cultures treated for two days with 1 μM mercuric chloride even though both lower and higher concentrations were stimulatory. In cultures treated for three days, a similar decrease in acetyl cholinesterase activity was also found with 1 μM mercuric chloride (Fig. 4). Since the two- and three-day treatment experiments were carried out at separate times, these results suggest that the small reduction observed at 1 μM might not be artifactual.

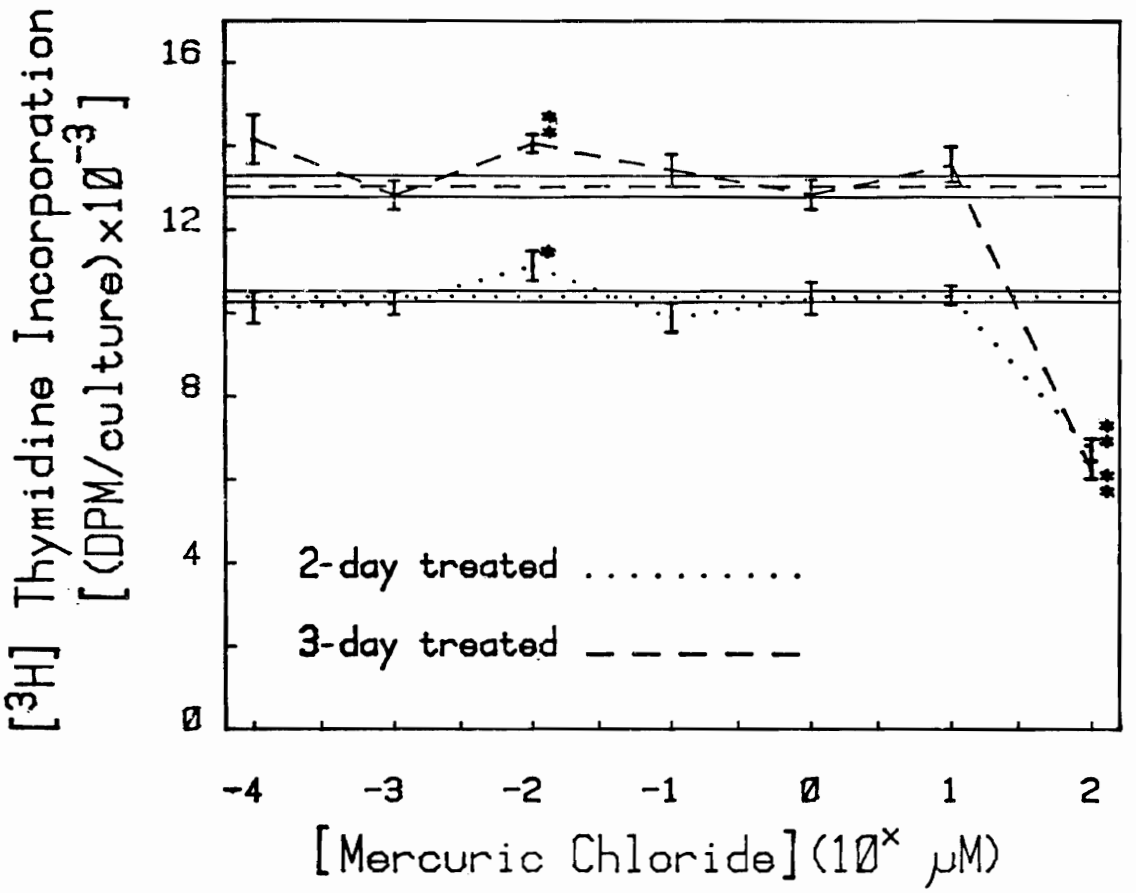
In contrast to the results obtained for cultures treated for two days, the level of acetyl cholinesterase activity was never elevated in mixed ganglion cell cultures treated with mercuric

chloride for three days (Fig. 4). However, significant decreases in acetyl cholinesterase activity were found at both 1 μM (vide supra) and at 100 μM concentrations of mercuric chloride.

$^{3}$H-Thymidine incorporation. The amount of $^{3}$H-thymidine incorporated into acid-precipitable macromolecules increased from 10,400 ± 700 DPM/culture (n = 6) after 72 hr to 13,000 ± 300 DPM/culture (n = 8) after 92 hr in vitro (see control values in Fig. 5 corresponding to two- and three-day treatment experiments, respectively). Since only the non-neuronal cells in these mixed cultures have the capacity to incorporate $^{3}$H-thymidine into DNA (McCarthy and Partlow, 1976a), incorporation of $^{3}$H-thymidine serves as a specific marker for non-neuronal cell multiplication. These results indicate that the amount of new DNA synthesized by the non-neuronal cells in untreated mixed cultures increased by 25% during the fourth day in vitro.

Very similar effects resulted from the treatment of mixed sympathetic cultures with mercuric chloride for either two or three days. Aside from a slight increase in $^{3}$H-thymidine incorporation which occurred at 10 nM in both two- and three-day treatment experiments, no effect was observed at any concentration of mercuric chloride from 0.0001 to 10 μM (Fig. 5). Thus, low concentrations of mercuric chloride have little if any effect on DNA synthesis by the non-neuronal cells. However, exposure of

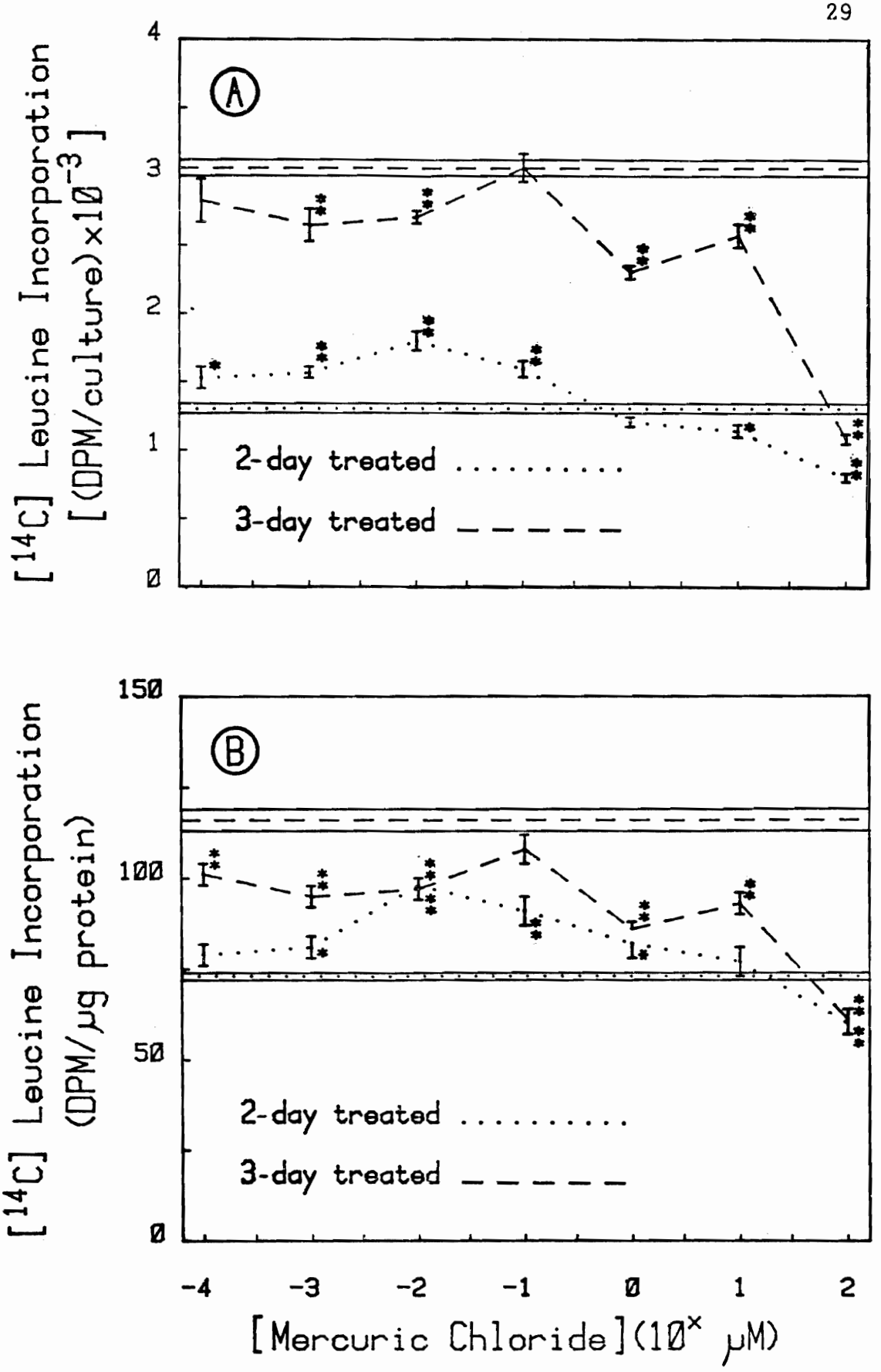
Figure 5. $^{3}$H-Thymidine incorporation by mixed sympathetic ganglion cell cultures exposed for either two or three days to various concentrations of mercuric chloride. Mixed cultures were prepared from the sympathetic ganglia of 11-day chick embryos, handled as described in Figure 2A, B, harvested by sonication, and assayed for $^{3}$H-thymidine incorporated into acid-precipitable macromolecules as described in Materials and Methods. All values represent means $\pm$ SE ($n = 4-8$). Values for untreated control cultures are shown as bars extending across the width of the graph for both two- and three-day treatment experiments. Means of treated cultures which differ significantly from that of the matched control are marked with a single asterisk (*) if $0.025 \geq p > 0.01$ and with two asterisks (**) if $p \leq 0.01$.


[3H] Thymidine Incorporation
[(DPM/culture)x10-3]
16
12
8
4
0
2-day treated .....
3-day treated _ _ _ _ _
-4
-3
-2
-1
0
1
2
[Mercuric Chloride](10x µM)

the mixed cultures to 100 μM mercuric chloride for two or three days reduced $^{3}$H-thymidine incorporation by 35% or 55%, respectively (Fig. 5). These reductions demonstrate that very high concentrations of mercuric chloride can inhibit the synthesis of new DNA by the non-neuronal cells present in these mixed cultures.

$^{14}$C-Leucine incorporation. The rate of synthesis of new protein in untreated mixed cultures was determined by measuring the amount of incorporated $^{14}$C-leucine. These results indicate that $^{14}$C-leucine incorporation into acid-precipitable macromolecules increased from 1,300 ± 30 DPM/culture (n = 6) for mixed cultures incubated for 72 hr to 3,060 ± 60 DPM/culture (n = 8) for cultures incubated for 92 hr in vitro (see control values in Fig. 6A corresponding to two- and three-day treatment experiments, respectively). Since both the neurons and non-neuronal cells in the mixed cultures synthesized protein, these values can also be properly expressed in terms of specific activity. When expressed in this manner, $^{14}$C-leucine incorporation increased from 73 ± 1 DPM/μg protein (n = 6) after 72 hr to 116 ± 3 DPM/μg protein (n = 8) after 92 hr in vitro (Fig. 6B). These results demonstrate that $^{14}$C-leucine incorporation per μg of protein increased by 59% in mixed sympathetic culture during the fourth day of incubation.

Figure 6. $^{14}$C-Leucine incorporation by mixed sympathetic ganglion cell cultures exposed for either two or three days to various concentrations of mercuric chloride. Mixed cultures were prepared from the sympathetic ganglia of 11-day chick embryos, handled as described in Figure 2A,B, harvested by sonication, and assayed for $^{14}$C-leucine incorporated into acid-precipitable macromolecules as described in Materials and Methods. All values represents means $\pm$ SE (n = 4-8). Values for untreated control cultures are shown as bars extending across the width of the graph for both two-and three-day treatment experiments. Means of treated cultures which differ significantly from that of the matched control are marked with a single asterisk (*) if $0.025 \geq p > 0.01$ and with two asterisk (**) if $p \leq 0.01$.


A
[14C] Leucine Incorporation
[(DPM/culture)x10-3]
4
3
2
1
0
2-day treated .....
3-day treated _ _ _ _ _
B
[14C] Leucine Incorporation
(DPM/µg protein)
150
100
50
0
2-day treated .....
3-day treated _ _ _ _ _
-4
-3
-2
-1
0
1
2
[Mercuric Chloride](10x µM)

In mixed cultures treated with mercuric chloride for two days, $^{14}$C-leucine incorporation was generally elevated at concentrations between 0.0001 and 1 μM when expressed in terms of either DPM/culture (Fig. 6A) or DPM/μg of protein (Fig. 6B). At 100 μM, $^{14}$C-leucine was significantly depressed when expressed either manner (Fig. 6A, B). These results demonstrate that low concentrations of mercuric chloride can stimulate $^{14}$C-leucine incorporation by the neurons and/or non-neuronal cells in the mixed cultures, while 100 μM mercuric chloride can inhibit protein synthesis by one or both of these cellular constituents.

In mixed cultures treated for three days, almost every concentration of mercuric chloride reduced $^{14}$C-leucine incorporation (Fig. 6). At a 100 μM concentration of mercuric chloride incorporation by mixed cultures was inhibited by either 65 or 47%, when expressed in terms of DPM/culture or DPM/μg of protein, respectively (Fig. 6). A comparison of the amounts of $^{14}$C-leucine incorporated by two- and three-day treatment cultures exposed to various concentrations of mercuric chloride demonstrates that the effects of the metallic salt are quite different after these two exposure periods (Fig. 6). The most obvious difference is that no concentration of mercuric chloride increased $^{14}$C-leucine incorporation by cultures treated for

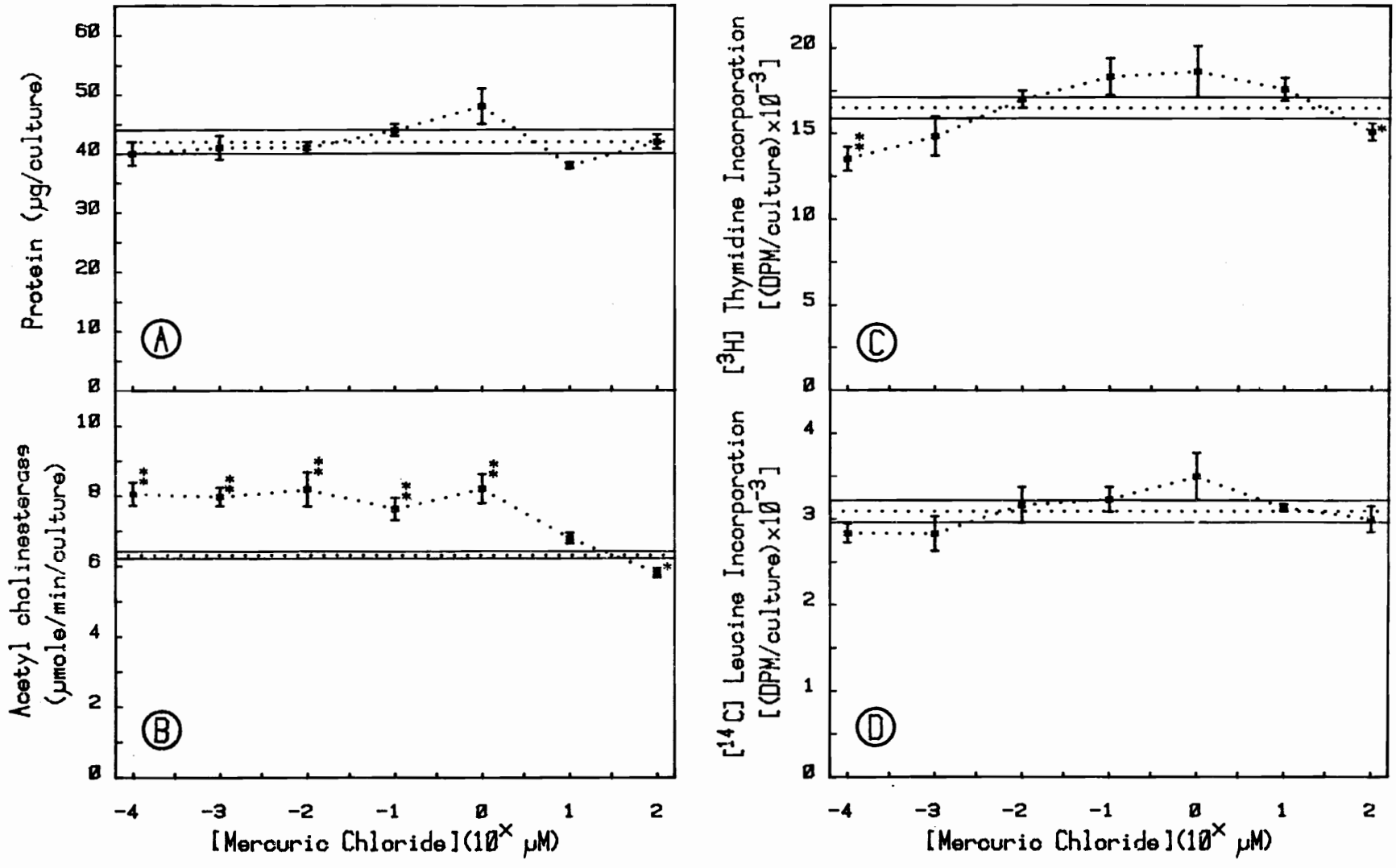
three days even though stimulation of protein synthesis was common in cultures treated for two days with low concentrations of this metallic salt (Fig. 6). Somewhat similar differences between two- and three-day treatment cultures were previously described for acetyl cholinesterase (Fig. 4) and, to a lesser extent, for total protein content (Fig. 3).

Effects of mercuric chloride on mixed sympathetic cultures treated for two days and allowed to recover for one additional day.

The mixed cultures used in this experiment were identical to two-day treatment cultures except that they were incubated for an additional 20 hr following removal of mercuric chloride from the medium (compare Figs. 1A and 1C). Thus, the cultures used in this recovery experiment were incubated for the same total period of time as three-day treatment cultures (compare Figs. 1B and 1C).

The results presented in Fig. 7 demonstrate that most of the effects of exposure to mercuric chloride for two days are completely reversed by a subsequent 20 hr incubation in medium lacking mercuric chloride. Thus, no significant difference was found at any concentration of mercuric chloride in either total protein content (Fig. 7A) or $^{14}$C-leucine

Figure 7. Effects of mercuric chloride on total protein content (A), acetyl cholinesterase activity (B), $^{3}H$-thymidine incorporation (C), and $^{14}C$-leucine incorporation (D) in mixed sympathetic ganglion cell cultures exposed for two days to various concentrations of mercuric chloride and then incubated for an additional 20 hr in medium lacking this metallic salt. Mixed cultures were prepared from the sympathetic ganglia of 11-day chick embryos and handled as described in Figure 2C. All values represent means $\pm$ SE ($n = 5$). Values for untreated control cultures are shown as bars extending across the width of the graph in panels A-D. Means of treated cultures which differ singificantly from that of the relevant control are marked with a single asterisk (*) if $0.025 \geq p > 0.01$ and with two asterisks (**) if $p \leq 0.01$.


Protein (µg/culture)
60
50
40
30
20
10
0
A
Acetyl cholinesterase
(µmole/min/culture)
10
8
6
4
2
0
B
-4
-3
-2
-1
0
1
2
[Mercuric Chloride](10^X µM)
[3H] Thymidine Incorporation
[(DPM/culture)x10^-3]
20
15
10
5
0
C
[14C] Leucine Incorporation
[(DPM/culture)x10^-3]
4
3
2
1
0
D
-4
-3
-2
-1
0
1
2
[Mercuric Chloride](10^X µM)

incorporation (Fig. 7D) even though both stimulatory and inhibitory effects were observed in two-day treatment cultures (Fig. 3 and 6). Similarly, mercuric chloride had little effect on $^{3}$H-thymidine incorporation by cultures incubated for an additional 20 hr in L-15$^{+}$ medium lacking the metallic salt (compare Figs. 5 and 7C).

In contrast to the results just described, the effects of a two-day exposure to mercuric chloride on acetyl cholinesterase activity persisted for at least 20 hr following removal of this metallic salt from the culture medium. Acetyl cholinesterase activity was stimulated by 16 to 38% at all concentrations from 0.0001 to 1 μM mercuric chloride (Fig. 7B). In addition, a small but significant reduction in acetyl cholinesterase activity was found at 100 μM (Fig. 1). These results are very similar to those obtained with two-day treatment cultures (Fig. 4) and suggest that new enzyme might have been synthesized during the two-day exposure to mercuric chloride.

Effects of mercuric chloride on both highly purified and mixed cultures of neuronal and non-neuronal cells.

Protein content. Relative values are given in Table 2 for the protein content of both mixed and highly purified cultures of neuronal and non-neuronal cells exposed for two days to various concentrations of mercuric chloride. A concentration of 0.01 μM

Table 2. Relative protein content of both highly purified and mixed cultures of sympathetic neurons and non-neuronal cells which were exposed to various concentrations of mercuric chloride for two days[+].

| $\frac{[HgCl_2]}{(\mu M)}$ | Relative Protein Content (% of control)[#] | | |
|---|---|---|---|
| | Mixed cultures | Non-neuronal cultures | Neuronal cultures |
| 0.01 | 100 ± 3 (4) | 100 ± 7 (6) | 100 ± 7 (6) |
| 1.0 | 83 ± 3 (6)** | 78 ± 6 (6)** | 105 ± 9 (5) |
| 100.0 | 72 ± 3 (6)** | 78 ± 7 (6)** | 110 ± 11 (6) |

[+] Both mixed and highly purified cultures were prepared from the sympathetic ganglia of 11-day chick embryos, handled as described in Figure 2, harvested by sonication, and assayed for total protein as described in Materials and Methods. All values represent means ± SE. Numbers of observations (n) are given in parentheses. Values which differ significantly from that of the matched control are marked with two asterisks (**) if $p \leq 0,01$.

[#] The total protein content of the mixed, non-neuronal, and neuronal cultures was 18 ± 0.3 (n=6), 9 ± 0.6 (n=5) and 5 ± 0.2 (n=5) μg/culture, respectively.

mercuric chloride did not affect the protein content in any of the three types of cultures (Table 2). In contrast, concentration of both 1 and 100 μM mercuric chloride significantly decreased the amounts of protein found in both mixed and non-neuronal cultures but had no effect on the protein content of the highly purified neuronal cultures (Table 2). Thus, it appears likely that the decrease in protein content observed in mixed cultures treated for two days with from 1 to 100 μM mercuric chloride (Fig. 3 and Table 2) is primarily due to a direct effect of the metallic salt on the non-neuronal cells present in the mixed cultures.

Acetyl cholinesterase activity. Relative values are given in Table 3 for acetyl cholinesterase activity in both mixed and highly purified neuronal cultures exposed for two days to various concentrations of mercuric chloride. Values are not given for the highly purified non-neuronal cultures as these were almost entirely devoid of contaminating neurons and therefore lacked detectable levels of acetyl cholinesterase. The results shown in Table 3 demonstrate that treatment with 0.01 μM mercuric chloride increased acetyl cholinesterase activity in both mixed and neuronal cultures to approximately the same extent. Thus, it appears that the increase in acetyl cholinesterase activity observed in mixed cultures treated for two days with low concentrations of mercuric chloride (Fig. 4 and Table 3) is probably due to a direct effect of the metallic salt on the

Table 3. Relative acetyl cholinesterase activity in both highly purified neuronal and mixed cultures of sympathetic ganglion cells which were exposed to various concentrations of mercuric chloride for two days [+].

| $\frac{[HgCl_2]}{(\mu M)}$ | Relative Acetyl cholinesterase activity (% if control) [#] | |
|---|---|---|
| | Mixed cultures | Neuronal cultures |
| 0.01 | 130 ± 5 (4) ** | 141 ± 8 (6) ** |
| 1.0 | 108 ± 7 (6) | ——— |
| 100.0 | 94 ± 7 (6) | 91 ± 8 (5) |

[+]Both mixed and highly purified neuronal cultures were prepared from the sympathetic ganglia of 11-day chick embryos, handled as described in Figure 2, harvested by sonication, and assayed for acetyl cholinesterase activity as described in Materials and Methods. All values represent means ± SE. Numbers of observations (n) are given in parentheses. Values which differ significantly from that of the matched control are marked with two asterisks (**) if $p \leq 0.01$.

[#]The acetyl cholinesterase activities of mixed and neuronal cultures were 3.9 ± 0.1 (n=6) and 1.42 ± 0.04 (n=5) μmoles/min/culture, respectively.

neurons present in the mixed cultures.

$^{3}$H-Thymidine incorporation. Relative values are given in Table 4 for the amounts of $^{3}$H-thymidine incorporated into both mixed cultures and highly purified non-neuronal cultures which had been exposed for two days to various concentrations of mercuric chloride. Treatment of both mixed and non-neuronal cultures with either 0.01 or 1 μM mercuric chloride had no significant effect on $^{3}$H-thymidine incorporation. In contrast, treatment with 100 μM mercuric chloride decreased incorporation of $^{3}$H-thymidine into mixed and non-neuronal cultures by 35% and 38%, respectively (Table 4). Thus, the direct effects of mercuric chloride on DNA synthesis by the non-neuronal cells could explain the actions of this metallic salt on $^{3}$H-thymidine incorporation by mixed cultures (Fig. 5 and Table 4).

Purified neuronal cultures incorporated approximately 19% as much $^{3}$H-thymidine as purified non-neuronal cultures having an equal number of cells (see the legend for Table 4). In such cultures, $^{3}$H-thymidine is primarily, if not entirely, incorporated by a small population of contaminating non-neuronal cells (≈1%) which divide exceedingly rapidly because of the presence of the vast excess of neurons (Wallace and Partlow, 1978; Hanson and Partlow, 1977). Treatment of such neuronal cultures for two days with either 0.01 or 1 μM mercuric chloride resulted in 15% and 19% reductions, respectively, in the amount of incorporated $^{3}$H-thymidine (Table 4). In

Table 4. Relative amounts of $^{3}$H-thymidine incorporated by both highly purified and mixed cultures of sympathetic neurons and non-neuronal cells which were exposed to various concentrations of mercuric chloride for two days[+].

| $[HgCl_2]$ (μM) | Relative $^{3}$H-Thymidine incorporation (% of control)[#] | | |
|---|---|---|---|
| | Mixed cultures | Non-neuronal cultures | Neuronal cultures |
| 0.01 | 107 ± 3 (4)* | 102 ± 6 (6) | 85 ± 6 (6)* |
| 1.0 | 100 ± 4 (6) | 104 ± 5 (6) | 81 ± 6 (6)** |
| 100.0 | 65 ± 3 (6)** | 62 ± 5 (6)** | 32 ± 2 (5)** |

[+]Both mixed and highly purified cultures were prepared from the sympathetic ganglia of 11-day chick embryos, handled as described in Figure 2, harvested by sonication, and assayed for $^{3}$H-thymidine incorporated into acid-precipitable macromolecules as described in Materials and Methods. All values represents means ± SE. Number of observations (n) are given in parentheses. Values which differ significantly from that of the matched control are marked with a single asterisk (*) if $0.025 \geq P \geq 0.01$ and with two asterisks (**) if $P \leq 0.01$.

[#]The amount of $^{3}$H-thymidine incorporated by mixed, neuronal, and non-neuronal cultures was 10,399 ± 133 (n=6), 2,154 ± 90 (n=5), and 11,119 ± 500 (n=6) DPM/culture, respectively.

contrast, neither mixed nor non-neuronal cultures were affected by either these concentrations (Table 4). Treatment of the neuronal cultures with 100 μM mercuric chloride resulted in a significantly greater reduction in $^{3}$H-thymidine incorporation than that seen with either mixed or non-neuronal cultures (68% vs 35-38%, respectively; Table 4). Thus, the non-neuronal cells present in the neuronal cultures were much more sensitive to inhibition of $^{3}$H-thymidine incorporation than either the mixed or highly purified non-neuronal cultures. These results suggest that the observed reduction in $^{3}$H-thymidine incorporation by contaminating non-neuronal cells might only partially be brought about by means of a direct action of the mercuric chloride on the non-neuronal cells. In addition, it seems possible that DNA synthesis by these highly stimulated non-neuronal cells might be partially inhibited by an indirect action of mercuric chloride on the neurons which are mitogenic for the non-neuronal cells (McCarthy and Partlow, 1976b; Wood and Bunge, 1975).

$^{14}$C-Leucine incorporation. Relative values are given in Table 5 for the incorporation of $^{14}$C-leucine by both mixed and highly purified cultures of neuronal and non-neuronal cells which had been exposed for two days to various concentrations of mercuric chloride. A mercuric chloride concentration of 0.01 μM increased $^{14}$C-leucine incorporation by mixed cultures but did not significantly alter incorporation by either the pure neuronal or non-neuronal cultures (Table

Table 5. Relative amounts of $^{14}$C-leucine incorporated by both highly purified and mixed cultures of sympathetic neurons and non-neuronal cells which were exposed to various concentrations of mercuric chloride for two days[+].

| $HgCl_2$ ($\mu M$) | Relative $^{14}$C-Leucine incorporation (% of control)[#] | | |
|---|---|---|---|
| | Mixed cultures | Non-neuronal cultures | Neuronal cultures |
| 0.01 | 138 ± 7 (4)** | 95 ± 5 (6) | 108 ± 8 (5) |
| 1.0 | 92 ± 4 (6) | 91 ± 5 (5) | 104 ± 8 (6) |
| 100.0 | 61 ± 3 (6)** | 76 ± 5 (6)** | 93 ± 6 (5) |

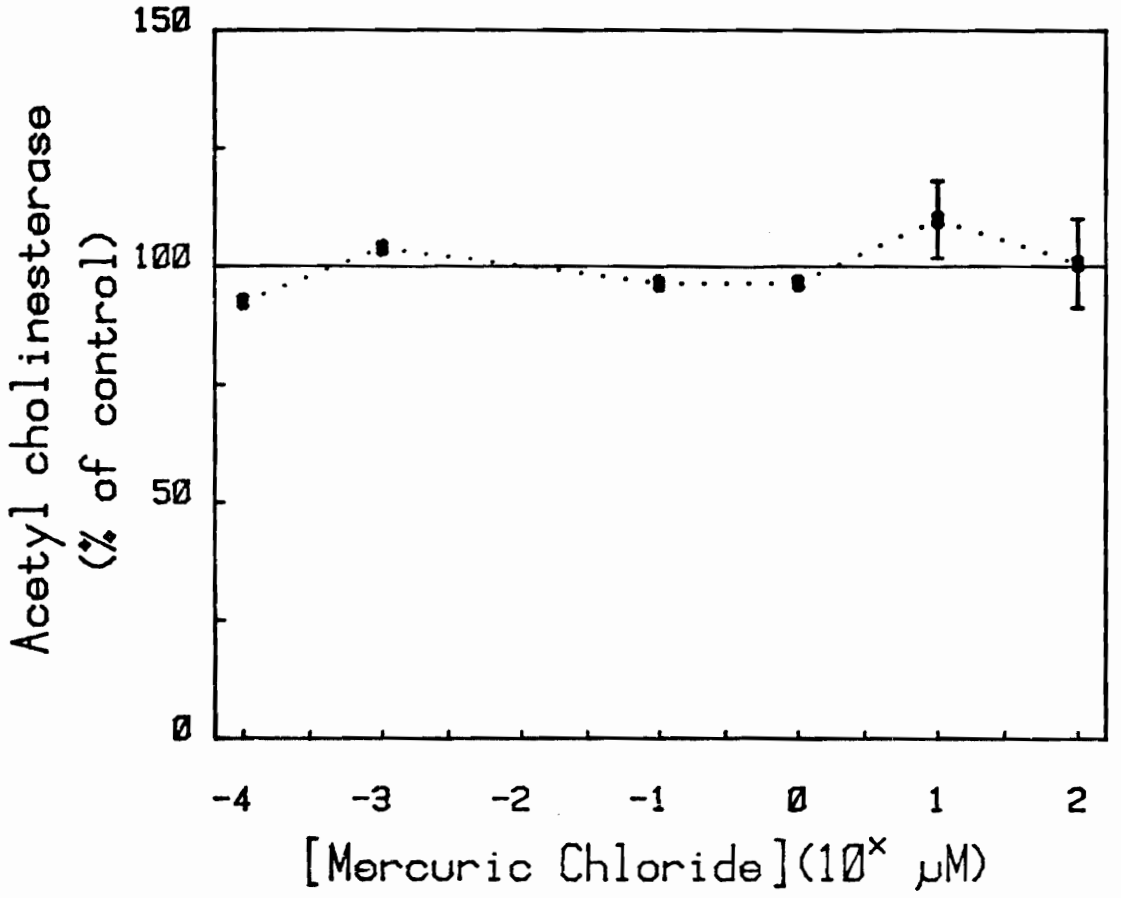
[+]Both mixed and highly purified cultures were prepared from the sympathetic ganglia of 11-day chick embryos, handled as described in Figure 2, harvested by sonication, and assayed for $^{14}$C-leucine incorporated into acid-precipitable macromolecules as described in Materials and Methods. All values represent means ± SE. Number of observations (n) are given in parentheses. Values which differ significantly from that of the matched control are marked with two asterisks (**) if $p \leq 0.01$.

[#]The amounts of $^{14}$C-leucine incorporated by mixed, neuronal, and non-neuronal cultures was 1,303 ± 35 (n = 6), 204 ± 9 ( n = 5 ) and 717 ± 27 ( n = 6 ) DPM/culture, respectively.

5). Treatment with 1 μM mercuric chloride had no significant effect on any of the three types of cultures (Table 5). In contrast, exposure to 100 μM mercuric chloride decreased incorporation of $^{14}$C-leucine into both mixed and pure non-neuronal cultures by 39% and 24%, respectively, but had no significant effect on incorporation by the pure neuronal cultures. Thus, the direct effect of this metallic salt on $^{14}$C-leucine incorporation by non-neuronal cells are probably sufficient to account for the inhibitory actions of 100 μM mercuric chloride on mixed cultures (Fig. 6 and Table 5). However, the stimulatory effect of 0.01 μM mercuric chloride on $^{14}$C-leucine incorporation by mixed cultures cannot be explained by direct action on either the neurons or the non-neuronal cells (Table 5). This observation suggests that 0.01 μM mercuric chloride might only stimulate $^{14}$C-leucine incorporation if both cell types are simultaneously present.

Effects of mercuric chloride on acetyl cholinesterase activity. Mixed sympathetic ganglion cell cultures were sonicated and incubated at room temperature for 1 hr in the presence or absence of various concentrations of mercuric chloride. None of the treated samples had levels of acetyl cholinesterase activity which were significantly different from control (Fig. 8). These results suggest that neither the stimulatory nor the inhibitory effects of mercuric chloride on acetyl cholinesterase activity (Fig. 4 and 7) can be explained by a direct action of the metallic salt on this enzyme molecule.

Figure 8. Lack of an effect of various concentrations of mercuric chloride on acetyl cholinesterase activity. Dense mixed cultures (475,000 cells/culture) were prepared from the sympathetic ganglia of 11-day chick embryos and incubated for 72 hr in vitro. Cells were harvested by sonication and then incubated with various concentrations of mercuric chloride for 1 hr at room temperature prior to assay for acetyl cholinesterase as described in Materials and Methods. Values represent single determinations at 0.0001 and 0.001 μM, means of a pair of observations at 0.1 and 1 μM, and means ± SE of three observations at 10 and 100 μM.


150
100
50
0
Acetyl cholinesterase
(% of control)
-4
-3
-2
-1
0
1
2
[Mercuric Chloride](10^x µM)

## DISCUSSION

The effects of mercuric chloride on the biochemistry of sympathetic neurons and non-neuronal cells in vitro have been shown to be dependent on both the concentration of the metallic salt and the time of exposure. Thus, a given biochemical variable might be stimulated by a low concentration of mercuric chloride but inhibited by a higher concentration. In addition, a concentration which causes stimulation after exposure for two days might cause inhibition after exposure for three days. Because of the complex nature of these effects, each biochemical variable will now be discussed separately.

Protein synthesis. Since both neurons and non-neuronal cells incorporate $^{14}$C-leucine into protein, results obtained with mixed cultures reflect the combined effects of mercuric chloride on both cell types. After two days of exposure, both total protein content (Fig. 2) and $^{14}$C-leucine incorporation (Fig. 5A) gradually declined as the concentration of mercuric chloride increased from 0.0001 μM to 100 μM. At very low concentrations (0.1-1.0 nM) both variables were significantly stimulated while at very high concentrations, both were inhibited (10-100 μM). The stimulatory and inhibitory effects observed after exposure for two days were shown to be completely reversible since no effects were found in cultures incubated for an

additional 20 hr in the absence of the metallic salt (Fig. 6A,D). After three days of exposure, $^{14}$C-leucine incorporation was inhibited at almost all concentrations while protein content was inhibited only at the highest concentration. Calculation of $^{14}$C-leucine incorporation in terms of specific activity (Fig. 5B) revealed that the observed changes in total protein were not sufficient to account for either the stimulatory or inhibitory effects of mercuric chloride.

Previous report have similarly shown that mercury compounds can either stimulate or inhibit protein synthesis depending on the precise experimental conditions. Both Yoshino et al. (1966) and Cavanagh and Chen (1971) have reported that administration of methyl mercury in vivo significantly reduced protein synthesis during the latent period before other neuropathological effects became apparent. In a recent study, Verity et al.(1977) confirmed the results of Yoshino et al. (1966) on the incorporation of amino acids by brain slices and further demonstrated that methyl mercury inhibited synaptosomal protein synthesis during the neurotoxic phase but not during the latent phase. In contrast, Brubaker et al. (1973) reported that methyl mercury caused an increase in the in vivo incorporation of labelled amino acids into protein.

The effects of a two-day exposure to mercuric chloride on protein synthesis by highly purified neuronal and non-neuronal cultures was examined in order to determine whether both cell types

responded similarly to this metallic salt. Protein content and $^{14}$C-leucine incorporation were inhibited to a similar extent in both mixed and non-neuronal cultures by 1 and 100 μM mercuric chloride (Tables 2 and 5). However, neither concentration had any significant effect on either biochemical variable in purified neuronal cultures (Tables 2 and 5). Thus, the inhibitory effect on protein synthesis observed in mixed cultures in micromolar concentrations probably resulted from a selective effect on non-neuronal cells.

Analysis of the stimulatory effects of 0.01 μM mercuric chloride on $^{14}$C-leucine incorporation by mixed cultures was not as straightforward. Thus, $^{14}$C-leucine incorporation was not significantly elevated in either purified neuronal or non-neuronal cultures even though incorporation by mixed cultures increased by nearly 40% (Table 5). These results suggest that stimulation of protein synthesis by mercuric chloride might in some way require the simultaneous presence of both cell types. It is conceivable that the mercuric chloride might alter some aspect of the dynamic interactive processes occurring between the neurons and non-neuronal cells which are loosely spoken of as neuronal-glial interactions.

$^{3}$H-Thymidine incorporation. Mercuric chloride had little effect on $^{3}$H-thymidine incorporation at concentrations less than 100 μM. Very small increases were observed in $^{3}$H-thymidine incorporation by mixed cultures after two or three days of exposure to 0.01 μM

mercuric chloride (7% and 8%, respectively; Fig. 4), but no similar effect was found in purified non-neuronal cultures (Table 4). $^{3}$H-thymidine incorporation by mixed cultures was markedly inhibited by exposure for two or three days to 100 μM mercuric chloride (35% and 55%, respectively). In purified non-neuronal cultures, exposure to 100 μM mercuric chloride for two days inhibited $^{3}$H-thymidine incorporation by 38% (Table 4). Thus, high concentrations of mercuric chloride probably primarily decreased $^{3}$H-thymidine incorporation in mixed cultures as a result of a direct inhibitory action on the non-neuronal cells.

Our observation that exposure to 100 μM mercuric chloride for two or three days markedly inhibited $^{3}$H-thymidine is in good agreement with in vitro studies on a variety of cell lines. Fisher (1976) reported that exposure to 13 μM mercuric chloride for seven days resulted in a 50% inhibition of the rate of cell division by a subline of L929 mouse fibroblasts. Potter and Matrone (1977) also reported a 50% reduction in cell multiplication by both 3T3 cells and Chang's liver cells exposed to $\approx$10 or $\approx$45 μM mercuric chloride, respectively, for four days.

Histopathological reports describing the brains of mercury-intoxicated humans and laboratory animals (Chang and Hartmann, 1972b; Charbonneau et al.,1976; Chang, 1977) have repeatedly pointed out that gliosis is a common response to heavy metal

poisoning. Nevertheless, the results presented in this paper suggest that glial cells probably do not undergo hypertrophy and hyperplasia (reactive gliosis) in response to a direct action of mercury. It therefore seems likely that the observed gliosis might be due to an indirect action of the heavy metal on the neurons. Lim et al. (1976) have demonstrated that brain homogenates contain materials which can stimulate the proliferation of glial cells. Hanson and Partlow (1978) have further shown that destruction of sympathetic neurons can release factors which are mitogenic for non-neuronal cells. Thus, the neuronal degeneration commonly observed in mercury-intoxicated brains could indirectly stimulate glial cell proliferation.

Results presented in this paper demonstrate that non-neuronal cells which are dividing exceedingly rapidly are more vunerable to inhibition by mercuric chloride than more quiescent non-neuronal cells. The division of the few non-neuronal cells present in purified neuronal cultures is greatly stimulated by the presence of the large excess of neurons (McCarthy and Partlow, 1976b; Hanson and Partlow, 1977). In such cultures, $^{3}$H-thymidine incorporation was reduced by up to 18% by exposure for two days to 0.01 or 1 μM mercuric chloride (Table 4). In contrast, incorporation by either mixed or purified non-neuronal cultures was unaffected by exposure to either concentration (Table 4). When cultures were exposed to 100 μM mercuric

chloride, $^{3}$H-thymidine incorporation by the neuronal cultures was nearly twice as inhibited as that by mixed or purified non-neuronal cultures (Table 4). These results suggest either (a) that non-neuronal cells which are dividing rapidly are inherently more sensitive to the direct effect of mercuric chloride or (b) that the greater reduction in $^{3}$H-thymidine incorporation resulted from additive inhibitory effects on both the neurons and the non-neuronal cells. Since McCarthy and Partlow (1976) have shown that contact with growing nerve fibers can increase $^{3}$H-thymidine incorporation by non-neuronal cells, mercuric chloride might have acted on the neurons to reduce the degree of stimulation of non-neuronal cell proliferation by either decreasing the rate and/or extent of nerve fiber outgrowth or by directly interfering with the neuronal-glial interactions which regulate the rate of non-neuronal cell proliferation. Inhibition of nerve fiber outgrowth by micromolar concentrations of mercuric chloride has been described in cultured embryonic chick dorsal root ganglia (Kasuya, 1972).

Acetyl cholinesterase activity. Exposure of mixed cultures for two days to concentrations of mercuric chloride less than 100 μM generally resulted in an increase in acetyl cholinesterase activity while exposure for three days either had no effect on acetyl cholinesterase or inhibited this enzyme (Fig. 3). Exposure of purified neuronal and mixed cultures to 0.01 μM mercuric chloride for two

days increased acetyl cholinesterase activity to a similar extent (Table 3). Thus, low concentrations of mercuric chloride probably increased acetyl cholinesterase activity in mixed cultures as a result of a direct stimulatory action on the sympathetic neurons.

For several reasons it appears likely that increased acetyl cholinesterase activity resulted from an increased synthesis of this neuronal enzyme. First, the stimulatory effects of mercuric chloride persisted during a subsequent 20 hr incubation in the absence of the heavy metal (Fig. 6). Second, concentrations of mercuric chloride which stimulated acetyl cholinesterase activity after exposure for two days were no longer stimulatory after exposure for three days (Fig. 3). Third, direct exposure of acetyl cholinesterase to mercuric chloride did not affect enzyme activity (Fig. 7). Thus, exposure to low concentrations of mercuric chloride for two days probably increased acetyl cholinesterase activity by stimulating the synthesis of at least some types of neuronal proteins.

Several previous reports have described the in vivo and in vitro effects of mercury on cholinesterase enzymes. Hrdina et al. (1976) reported that chronic exposure of rats to methyl mercury had no significant effect on the levels of acetyl cholinesterase in the cerebral cortex. In contrast, Christensen (1975) reported that the level of acetyl cholinesterase was reduced by 36% in baby brook trout which had been chronically exposed to water containing 0.005

μM methyl mercury. Goldstein and Doherty (1951) have studied the direct effects of mercuric chloride on plasma cholinesterase. They concluded that mercuric chloride could inactivate cholinesterase by two independent mechanisms. One of these mechanisms involved reversible binding at the active site while the other was characterized by irreversible combination at some other site on enzyme. Thus, these results support our finding that high concentrations of mercuric chloride can reduce acetyl cholinesterase activity. In contrast, no support can be found from previous studies for our demonstration that very low concentrations of mercuric chloride can increase acetyl cholinesterase activity.

General Conclusions.

Relative sensitivity of neurons and non-neuronal cells.

Neurons in both purified neuronal and mixed cultures appeared to be far more sensitive to mercuric chloride than the non-neuronal cells in either purified non-neuronal or mixed cultures. Thus, concentrations as low as 0.001 μM mercuric chloride significantly increased activity of the neuronal enzyme acetyl cholinesterase (Fig. 3) by a mechanism which appeared to involve new protein synthesis (vide supra). Furthermore, the inhibition of $^{3}$H-thymidine incorporation into the few contaminating non-neuronal cells present in neuronal cultures suggests that low concentrations of mercuric chloride primarily act

on the neurons to reduce the rate of non-neuronal cell proliferation (vide supra). It is tentatively assumed that exposure to low concentrations of mercuric chloride also increased both total protein content (Fig. 2) and $^{14}$C-leucine incorporation in mixed cultures by direct action on the neurons. In contrast, concentrations of mercuric chloride below 1 μM generally had no effect either on $^{3}$H-thymidine incorporation by mixed cultures (Fig. 4) or on total protein content, $^{14}$C-leucine incorporation, or $^{3}$H-thymidine incorporation in purified non-neuronal cultures (Tables 2, 4, and 5). Thus, available biochemical indices suggest that the neurons rather than the glial cells might be the primary target of mercuric chloride in the nervous system. A recent study by Sobkowicz and Murray supports this contention (Murray, 1971). These investigators studied the morphological and histochemical effects of heavy metals on organized primary cultures derived from dorsal root ganglia and cerebellar tissues. They reported that mercury was neurotoxic at exceedingly low concentrations and that it exerted its primary action on the neurons rather than the glial cells.

Direct vs. indirect effects on neurons and non-neuronal cells. Availability of pure cultures of neurons and non-neuronal cells has made it is possible to separate out some of the direct and indirect effects of mercuric chloride. Thus, the observed reductions in total protein content, $^{14}$C-leucine incorporation, and $^{3}$H-thymidine incor-

poration in mixed cultures exposed to high concentrations of mercuric chloride were clearly due to a direct action of the heavy metal on the non-neuronal cells (Table 2, 4, and 5). In addition, the observed increase in acetyl cholinesterase activity in the mixed cultures was clearly due to a direct action on the sympathetic neurons (Table 3). In contrast, certain effects caused by exposure of mixed cultures to mercuric chloride could not be reproduced in either purified neuronal or non-neuronal cultures. Thus, exposure for two days to low concentrations of mercuric chloride increased $^{14}$C-leucine incorporation by mixed cultures but had no significant effect on either purified neuronal or non-neuronal cultures (Table 4). This suggest that the stimulatory effect on protein synthesis by mixed cultures was indirect rather than direct and that it required the simultaneous presence of both cell types in order to be observed. Gliosis is another effect of heavy metal intoxication that appears to be indirect. Thus, mercuric chloride did not directly stimulate glial cell proliferation in either purified non-neuronal or mixed cultures (Fig. 4 and Table 4). It is proposed that gliosis in animals chronically exposed to mercury compound might result from a primary degeneration effect on the neurons which might lead to release of substances that are mitogenic for glia (Hanson and Partlow, 1978; vide supra).

Data presented in this paper clearly demonstrated that mercuric chloride has significant direct effects on the neurons and

glia which compose the nervous system. Thus, effects of mercuric chloride on brain biochemistry are at very least only partially due to indirect effects such as impairment of the blood-brain barrier (Chang and Hartmann, 1972c; Ware et al.,1974)

Concentrations of mercuric chloride. The lowest concentration of mercury compound previously reported to have any significant effect on any aspect of brain biochemistry was 0.005 μM (Christensen, 1975). Normal concentrations of mercury in human brain tissues have been variously reported as 0.03 μM (Hilmy et al., 1976), 1.2 μM (Olszewski et al., 1974) and 2.5 μM (Takeuchi et al., 1962). Brain levels in patients suffering from lethal mercury intoxication have been reported as 22.5-50 μM (Hilmy et al., 1976) and 0.5-124 μM (Takeuchi et al., 1962). The concentrations of mercuric chloride used in the present study were selected in order to bracket this concentration range.

All inhibitory effects reported in this paper occurred at concentrations of mercuric chloride between 1 and 100 μM. Thus, inhibition of protein synthesis, DNA synthesis, and/or acetyl cholinesterase might reasonably be encountered in the brains of individuals exposed to toxic concentrations of mercury.

In contrast, the stimulatory effects of acute exposure to very low concentrations of mercuric chloride on protein synthesis and acetyl cholinesterase activity would not be expected in mercury-

intoxicated nervous tissues because (a) these effects are transient and disappear with continued exposure (compare data for two and three three days of exposure in Fig. 2, 3, and 5), and (b) the concentrations which stimulate these biochemical variables involved are generally lower than those found in the brains of normal individuals at autopsy (vide supra).

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